Supporting information

Supplemental Methods

Isolation and culture of primary rat MSCs. We obtained MSCs from bone marrow aspirates of femurs and tibias from 4-week-old male Sprague-Dawley rats (approximately 100 g) with 10 ml of DMEM-low glucose medium supplemented with 10% FBS and 1% antibiotic-penicillin/streptomycin solution (Invitrogen). Collected media were centrifuged at 1,600 rpm, 5 min, resuspended in MSC medium, and separated by Percoll density gradient centrifugation with Ficoll-Paque^{TM} PLUS (GE Healthcare Life Sciences) at 1,600 rpm, 30 min. Bone marrow mononuclear cells were recovered from the middle interface after centrifugation, washed twice with PBS, resuspended in 10% FBS-DMEM, and plated in 100 cm^2 flasks. Culture conditions were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 72 h, non-adherent cells were discarded by tapping the flask, and adherent cells were thoroughly washed twice with PBS. Fresh MesenPRO RS^{TM} Medium (Invitrogen) was added, and then replaced every 3 d for approximately 10 d for stable multipotentiality. To further purify the MSCs, the Isolex Magnetic Cell Selection System (Baxter Healthcare Corporation) was used. Briefly, MSCs were incubated with Dynabeads M-450 coated with anti-CD34 monoclonal antibody. A magnetic field was applied to the chamber and the CD34+ cell-bead complexes were separated magnetically from the remaining cell suspension with the CD34- fraction being further cultured. The cells were harvested after incubation with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C, replated in 1 x 10^5/100-cm^2 plates, and again grown for approximately 10 days.

Isolation and culture of neonatal rat ventricular cardiomyocytes. Cardiomyocytes were prepared from Sprague-Dawley neonatal rat hearts. The isolated heart tissues

were washed with Dulbecco's phosphate-buffered saline solution (pH 7.4 Gibco BRL, NY, USA) in order to deplete red blood cells. Hearts were minced with micro-dissecting scissors until the pieces were approximately 0.5 mm^3 and then treated with 4 ml of collagenase II (1.4 mg/ml, 270 units/mg, Gibco BRL) for 5 min in a 37°C humidified chamber. The supernatant was then removed and washed with 10% FBS DMEM. The cells were resuspended in equal volumes of fresh medium containing 10% FBS. The remained tissue was treated with fresh collagenase II solution for an additional 5 min. The incubation procedure was repeated until the tissue was totally digested. The resulting supernatant was centrifuged at 2000 rpm for 2 min at room temperature. The cell pellet was resuspended in 5 ml of cell culture medium and plated on a culture dish for at least 2 hrs at 37°C in 5% CO_2 incubator. Under such conditions, the adhered cells are fibroblast and non-adherent cells are cardiomyocytes. Unattached cardiomyocytes were replated on 100 mm culture dishes (5×10^5cell/ml) and incubated in α-MEM supplemented with 10% FBS in a CO_2 incubator at 37°C. To reduce fibroblast contamination, we used α-MEM with 0.1mM 5-bromo-2'-deoxyuridine (Brd-U) (Sigma, MO, USA).

Sandwich ELISA. Capture antibody of 100 ng was bound to a polyvinylchloride (PVC) microtiter high binding plate (96 well) at 4°C, overnight. The plate was washed twice with PBS, and we blocked capture antibody with 5% BSA in PBS overnight in a humid atmosphere at room temperature. After washing the plate twice with PBS, 5 ug of cell lysate were added to each well with blocking buffer, and the plate was incubated for 1.5 h at 37°C. The plate was washed four times with PBS containing 0.02% Tween-20. Following the addition of detector antibody, the plate was incubated for 2 h at room temperature in a humid atmosphere, and was washed four times with PBS containing

0.02% Tween-20. The plate was then incubated again with peroxidase-conjugated secondary antibody diluted 1:1000 with 3% BSA for 1.5 h at 37°C, and then washed four times with PBS containing 0.02% Tween-20. Finally, 100 μl of tetramethylbenzidine (TMB) solution (Sigma) was poured as substrate. After 10 min, 25 μl of 0.1 M H_2SO_4 was added to stop the reaction, and then absorbance was determined immediately at 450 nm on an ELISA plate reader (Bio-Rad).

Induction of myocardial infarction and transplantation. All experimental procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine, and performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Myocardial infarction was produced in 8-week-old Sprague-Dawley male rats (250 g) by surgical occlusion of the left anterior descending (LAD) coronary artery, according to previously described procedures (8). Briefly, after induction of anesthesia with zoletil (20 mg/kg) and xylazine (5 mg/kg), we cut the third and fourth ribs to open the chest, and the heart was exteriorized through the intercostal space. The heart was exposed through a 2-cm left lateral thoracotomy. The pericardium was incised and a 6-0 silk suture (Johnson & Johnson) was placed around the proximal portion of the left coronary artery, beneath the left atrial appendage. Ligature ends were passed through a small length of plastic tube to form a snare. For coronary artery occlusion, the snare was pressed onto the surface of the heart directly above the coronary artery and a hemostat applied to the snare. After 60 min of occlusion, the hemostat was removed and the snare released for reperfusion, with the ligature left loose on the surface of the heart. For transplantation, control MSCs treated with DMSO and PMA-treated cells on day 9 were suspended in 30 μl of PBS (1×10^6 cells), and injected at three injection sites into anterior and lateral

aspects of the viable myocardium bordering the infarction using a Hamilton syringe (Hamilton Co.) with a 30-gauge needle. Sham-animals were injected with cell-free PBS. Throughout the operation, animals were ventilated with 95% O_2 and 5% CO_2 using a Harvard ventilator. All rats died during the procedure or immediately after cell implantation were excluded for mortalities, and autopsies were performed with all suddenly died rats to confirm that the animals were not died with cardiac rupture. We could confirm that rats were not died with cardiac rupture by autopsies, however, we did not determine the specific mode of sudden death of all died rats in this study.

To label cells with GFP, cells were transduced by adding purified LentiVs harboring GFP gene (Macrogen) to the cells in the presence of 8 μg/ml of polybrene at MOIs of 50 in 250 μl of medium at 1 day before transplantation. The cells were washed twice and replaced with fresh DMEM at 20 hours after transduction. DAPI to identify viable cells, sterile DAPI solution was added into the culture medium on the day of implantation at a final concentration of 50 μg/ml. The dye was allowed to remain in the culture dishes for 30 min. The cells were rinsed six times in PBS to remove all excess and unbound DAPI. Labeled cells were then detached with 0.25% (wt/vol.) trypsin and suspended in PBS for grafting. Histological analyses such as, TTC staining, Masson's trichrome, TUNEL assay, H&E staining, immunostaining, and immunofluorescence staining, electrical analysis such as,optical mapping, and functional analysis such as, millar catheterization were performed at 7, 11, and 21 days, respectively, after transplantation.

Histological analysis. Animals receiving transplants were sacrificed at 7 days after implantation, and their hearts were excised. Hearts were perfusion-fixed with 10% (vol/vol) neutral buffered formaldehyde for 24 h, transversely sectioned into four comparably thick sections, and embedded in paraffin using routine methods. Sections

of 5-μm thicknesses were mounted onto gelatin-coated glass slides to ensure that different stains could be used on successive sections of tissue cut through the implantation area. Histological analysis was performed using the manufacturer's instructions (Vector Laboratories). In brief, tissue sections were deparaffinized, rehydrated, and rinsed with PBS. Antigen retrieval was performed by microwaving for ten min with 10 mM sodium citrate (pH 6.0). Sections were incubated in 3% H_2O_2 to quench any endogenous peroxidase activity. Samples were blocked in 2.5% normal horse serum and incubated in primary antibody (CD31, collagen I, fibronectin, - smooth muscle actin). Biotinylated pan-specific universal secondary antibody and streptavidin/peroxidase complex reagent were used for the heart sections, which were stained with antibody using a DAB substrate kit. Counterstaining was with 1% methyl green, and dehydration progressed with 100% N-butanol, ethanol and xylene. Other serial sections were analyzed with rabbit anti-connexin 43. FITC-conjugated goat anti-rabbit IgG was used as a secondary antibody. TUNEL assay was operated as below. Sections were pretreated with 3.0% H_2O_2, subjected to TdT enzyme for 37°C for 1 h, and incubated in digoxigenin-conjugated nucleotide substrate at 37°C for 30 min. Nuclei exhibiting DNA fragmentation were visualized by DAB. Apoptotic index was calculated by a ratio of TUNEL positive cells to whole cells in the field. All images were produced using an excitation filter under reflected light fluorescence microscopy and were transferred to a computer equipped with MetaMorph software ver. 4.6 (Universal Imaging Corp). To detect interstitial fibrosis areas within the differentiated cell grafts, sections were analyzed by Masson’s trichrome staining and were measured with Image J 1.40g software. Hematoxylin and eosin (HE) staining and TUNEL assay were performed to measure morphological changes and inflammatory cell infiltration, which were carefully analyzed by three persons blinded to the treatments. For each group, six

slices were prepared and ten different regions were observed per slice.

Western blot. Cells were washed once with PBS, and lysed for about 20 min with lysis buffer (Cell Signaling Technology) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysate was centrifuged at 12,000 g for 10 min and the supernatant was reserved. Protein concentrations were determined using a Bradford Protein Assay Kit (Bio-Rad). Quantitative proteins were separated in a 12% sodium dodecyl sulfate-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% nonfat dried milk for 1 h at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibody (ERK and p-ERK; Santa Cruz Biotechnology) overnight at 4°C. The membrane was washed three times with TBS-T for 10 min, and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, bands were detected by enhanced chemiluminescence reagent (GE Healthcare Life Sciences). The band intensities were quantified using Image J 1.40g software (NIH).

RT-PCR. The expression levels of various genes were analyzed by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted with 500 μl Tri-reagent per 60 mm plate (Sigma). We poured 100 μl of chloroform over the Tri-reagent and vortexed each sample for about 10 secs. The sample was then centrifuged at 12,000 g and 4°C for 15 min. After this, three layers appeared in the tube, and the upper transparent layer was collected to new tubes. Thereafter, 250 μl of 2-propanol

was added to the sample, and the mixture was again vortexed for about 30 sec, followed by centrifugation at 12,000 g and 4°C for 10 min. Next, the supernatant was discarded, and the pellet was washed with 75% ethanol (Duksan) mixed with diethylpyrocarbonate (DEPC; Sigma) water. At this point, centrifugation was done at 7,500 g and 4°C for 5 min, and the pellet was dried at room temperature for about 7 min. Finally, 30 μl of nuclease free water was added. The quality and quantity of the RNA were detected with the OD_{260}/OD_{280} value using a DU 640 spectrophotometer (Eppendorf). Complementary DNA was generated with the Reverse Transcription System (Promega) according to the manufacturer's instructions. One μg of total RNA was reverse-transcribed in a 20 μl reaction mix containing 5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH 9.0 at 25°C), 50 mmol/L KCl, 0.1% Triton X-100, 1 mmol/L dNTPs, 20 U of RNase inhibitor, 0.5 μg oligo-$(dT)_{15}$ primer, and 10 U of reverse transcriptase for 15 min at 42°C, and the reaction was terminated by heating at 99°C for 5 min. The PCR mix contained 10 pmol/μl of each primer, together with 200 mM Tri-HCl (pH 8.8), 100 mM KCl, 1.5 mmol/L $MgSO_4$, 1% Triton X-100, 0.1 mM dNTPs, and 1.25 U of Taq polymerase in a total volume of 25 μl. PCR conditions consisted of a cycle of denaturing at 94°C for 3 min, and followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 49°C for 30 sec, and extension at 72°C for 2 min before a final extension at 72°C for 10 min. RT-PCR products were separated by electrophoresis in a 1.2% agarose gel, and visualized after staining with ethidium bromide.

Immunofluorescence. Immunocytochemical characterization of MSCs is demonstrated below. Cells were cultured in a 4-well slide chamber, washed with PBS, and incubated in 1% paraformaldehyde solution for 10 min. The cells were then washed twice with PBS before being permeabilized in 0.1% Triton X-100 for 7 min.

After this, the cells were blocked for 1 h (blocking solution: 2% bovine serum albumin and 10% horse serum in PBS) and then stained with the appropriate antibodies. FITC-conjugated mouse, rabbit, and goat (Jackson Immunoresearch Laboratories) secondary antibodies were used. Immunofluorescence was detected with confocal microscopy (Carl Zeiss).

Detection of cytokines. The expression levels of cytokines within the area injected with cells were determined using the rat cytokine array 3.1 (RayBiotech) according to the manufacturer's instructions. We recommend using 1 x cell lysis buffer to extract proteins from tissues. After extraction, samples were spun down, and the supernatant was saved. Protein concentration was measured, and samples were diluted in 2 x cell lysis buffer with H_2O. Samples were mixed with 2 ml 1 x blocking buffer and incubated at room temperature for 30 min to block membranes. Membranes were incubated with 1 ml of sample at room temperature for 1-2 h, decanted, and washed 3 times with 2 ml of 1X wash buffer I at room temperature for 5 min with shaking. Blots were washed twice with 2 ml of 1 x wash buffer II at room temperature for 5 min with shaking. After the addition of 100 µl of biotin-conjugated anti-cytokines with 1 x blocking buffer, blots were mixed gently and incubated at room temperature for 1-2 h. Wash buffer I and II were poured 5 times onto the blots, and 1,000 fold diluted HRP-conjugated streptavidin was injected to each membrane at room temperature for 2 h. Wash buffer I and II were poured 5 times onto the blots, and 250 µl of 1 x detection buffer C and 250 µl of 1 x detection buffer D were added and then incubated at room temperature for 2 min. Finally, arrays were exposed to x-ray film, and signal was detected using a chemiluminescence imaging system.

Optical mapping. Adult male rats (250-300 g) were injected with zoletil (20 mg/kg) and xylazine (5 mg/kg) plus heparin (200 U/kg). Eleven days after cell transplantation, the heart was excised and retrogradely perfused through the aorta with Tyrode's solution (in mM): 125 NaCl, 24 $NaHCO_3$, 1.0 $MgCl_2$, 4.0 KCl, 1.2 NaH_2PO_4, 5 dextrose, 25 mannitol, 1.25 $CaCl_2$, at pH 7.4, gassed with 95% O_2 and 5% CO_2. The temperature was maintained at 37.0 ± 0.2℃, and perfusion pressure was adjusted to ~60 mm Hg with a peristaltic pump. Hearts were stained with a voltage sensitive dye, di-4 ANEPPS (Invitrogen), 2.5 μl stock solution (1 mg/ml dimethyl sulfoxide, DMSO) delivered through the bubble trap, above the aortic cannula. ECGs were continuously monitored using a Biopac System (BIOPAC Systems Inc.). The hearts were placed in a chamber to maintain a constant temperature and to reduce movement artifacts, and 5 μM blebbistatin was added to the perfusate. Conduction velocity within the infarct or normal regions was measured under point stimulation at a cycle length of 280 ms for 20 beats. Vulnerability to VT was tested by ventricular burst stimulations performed at a stimulation cycle length (S1S1-CL) starting at 300 ms with 10-ms stepwise reductions down to 100 ms. Fluorescence images from the anterior surface of the heart were focused on a CCD camera (128 x 128 pixels, Dalsa Inc.), and the field of view was set to 1.0 x 1.0 cm^2 with a spatial resolution of 78 x 78 μm^2. The sampling rate was set to 420 frames/sec, and the data was analyzed with custom built software using Matlab (Mathworks, Natick). Activation and repolarization time-points at each site were determined from $(dF/dt)_{max}$ and $(d^2F/dt^2)_{max}$ and were shown to coincide with ~97% repolarization to baseline and recovery from refractoriness. Data were filtered with a Gaussian low pass filter (3 x 3 pixels) in spatial domain, and first/second order derivatives (dF/dt, d^2F/dt^2) were calculated using a polynomial filter (third order, 13 points) in the temporal domain. Local conduction velocity vectors were estimated from

each pixel's seven nearest neighbors with respect to the activation time of its temporal wave (1). The distribution of local CV could have high frequency components, such as near the pacing location, motion artifacts caused by transmural activity, and collisions between waves. For improving the SNR (signal to noise ratio) of local CV, the local CV was spatially filtered using a 7 x 7 nearest neighbor Gaussian convolution kernel which suppressed the acute change of its magnitude using log-transformation. CV map of anterior surface of heart focused by CCD camera was derived from local CV.

Surface ECG. A surface 6-lead ECG (lead II is shown in figures) was obtained in control, sham, MSC, and modified MSC-engrafted rats for 5 min. R–R intervals, PR intervals, QRS durations, QT, and corrected QT interval were measured by successive evaluation as described previously. The measured surface ECG parameters are described in table S1. All data were acquired at 1 ksps (kilo-sample per second) using a Bard stamp amplifier System (C.R. Bard Inc.).

Systemic administration of isoproterenol. Isoproterenol (2mg/kg) was injected intraperitoneally after surface 6-lead electrocardiography leads (limbs). The number of episodes of premature ventricular contractions during 15 minutes immediately after injection was calculated.

Left ventricular catheterization. For invasive hemodynamics, left ventricular catheterization was performed at 21 days after infarction. A Millar Mikro-tip 2 F pressure transducer (model SPR-838, Millar Instruments, Houston, TX, USA) was introduced into the left ventricle via the right carotid artery under zoletil (20 mg/kg) and xylazine (5 mg/kg) anesthesia. Real time pressure-volume loops were recorded by a

blinded investigator and all data were analyzed off-line with PVAN 3.5 software (Millar).

Statistical analysis. Data are expressed as means ±SE. The statistical analyses of two groups were estimated using Student's t-test. Examination of more than two groups was conducted using one-way ANOVA with a Bonferroni correction. p values < 0.05 were considered significant. For the Kaplan-Meier method with the log-rank test, a p value <0.05 was considered significant.

1. Salama, G, Kanai, A & Efimov, IR (1998) Subthreshold stimulation of Purkinje fibers interrupts ventricular tachycardia in intact hearts. Experimental study with voltage-sensitive dyes and imaging techniques. *Circulation Research* 74(4): 604–619.

Supplemental Figures

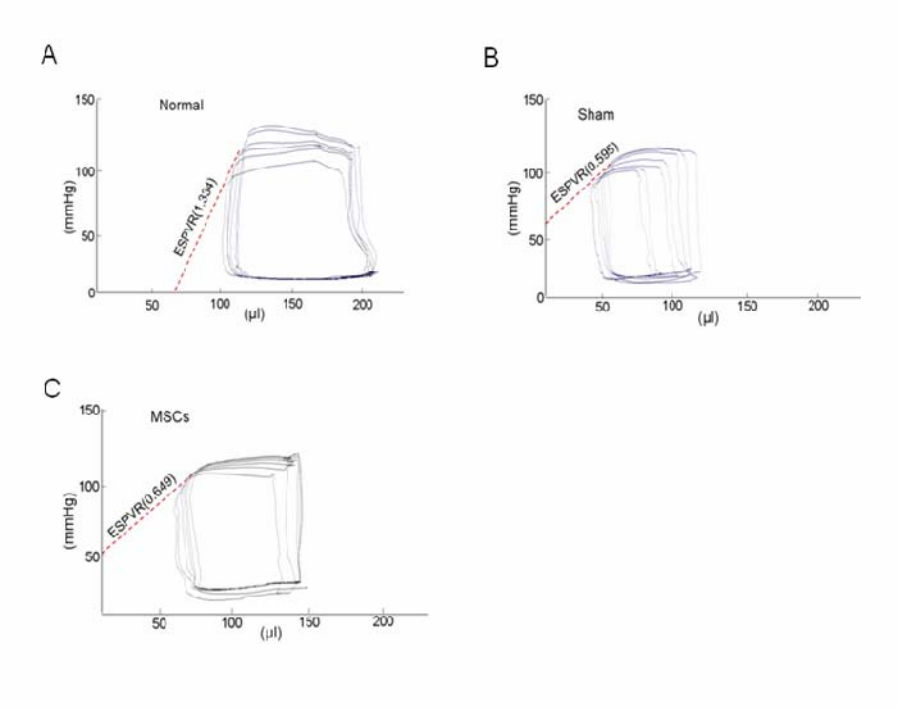

Fig. S1. Representative left ventricular pressure volume loops in normal (A) and sham- (B), and MSC- (C) injected rats. ESPVR during preload reduction is indicated by the dashed line.

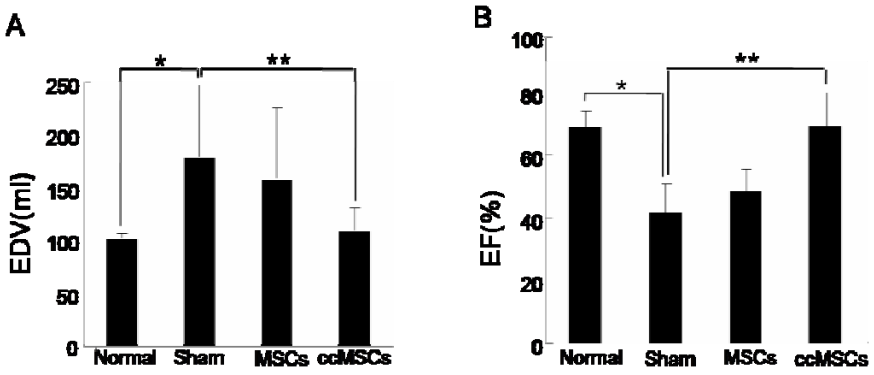

Fig. S2. Left ventricular volumes (A) and ejection fraction (B) measured by left ventricular catheterization in normal, sham-, MSC-, and the ccMSC-injected rats.

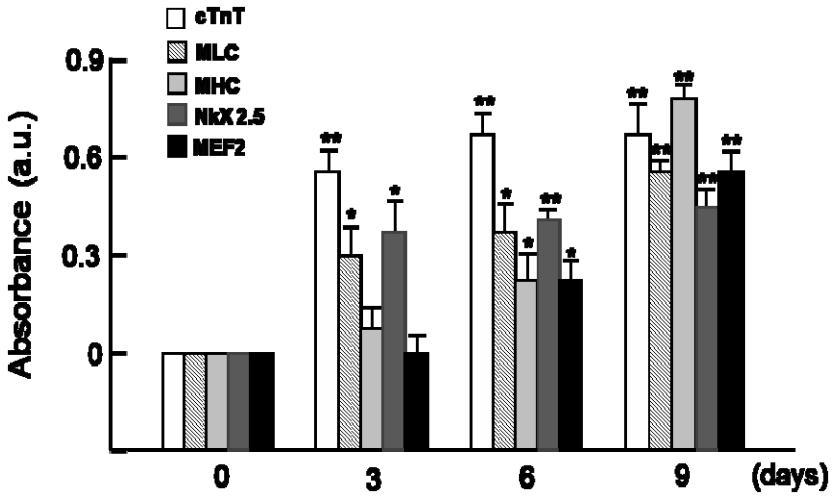

Fig. S3. The altered expression levels of cardiac specific markers, cTnT, MLC, MHC, MEF2, and NkX2.5 in the ccMSCs. Values were normalized against values from MSC control and are the mean ± SEM of three independent experiments.

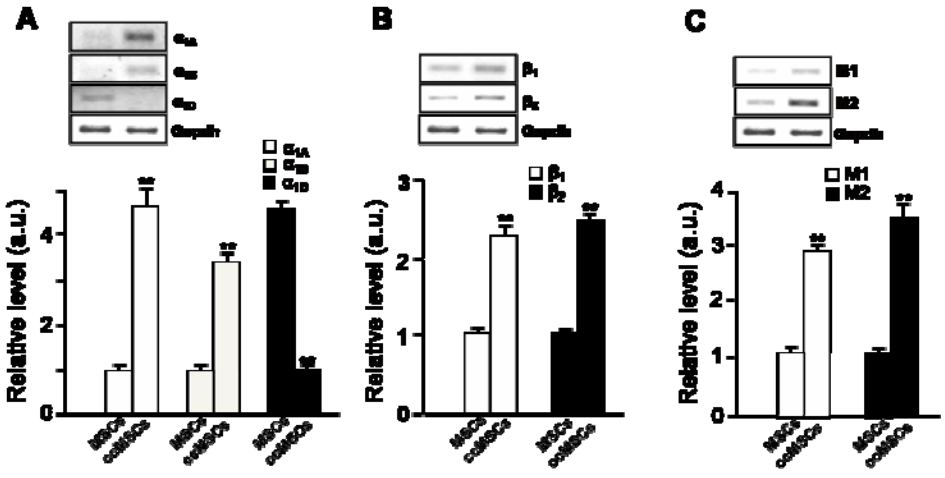

Fig. S4. The altered expression levels of mRNA of functional receptors, α-adrenergic receptors (A), β-adrenergic receptors (B), and muscarinic receptors (C), in normal MSCs and the ccMSCs. Values are the mean ± SEM of three independent experiments.

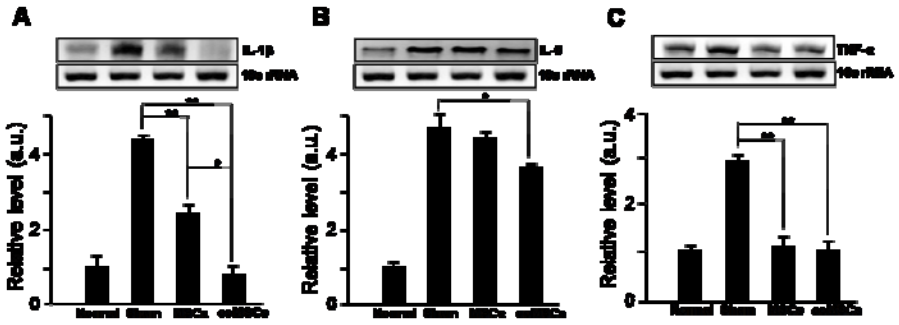

Fig. S5. The altered expression levels of mRNA of inflammatory cytokines in the infarcted region in normal, sham-injected, MSC-, and the ccMSC-engrafted hearts. Expression levels of (A) Interleukin-1 beta (IL-1β), (B) Interleukin-6 (IL-6) and (C) Tumor necrosis factor-alpha (TNF-α) were estimated in ischemic heart using RT-PCR. Values are the mean ± SEM of three independent experiments. **p<0.01.

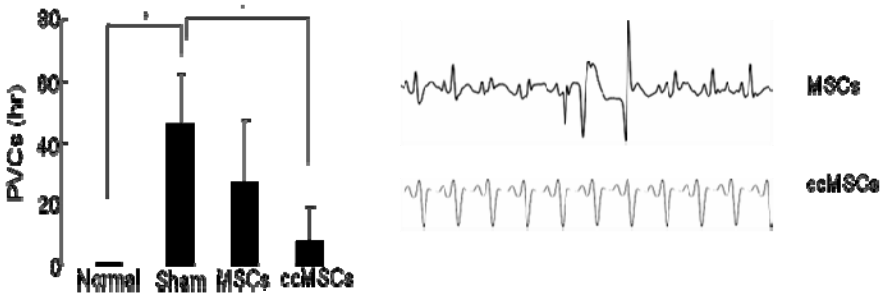

Fig. S6. Numbers of premature ventricular contractions (PVCs) during intraperitoneal isoproterenol injection recorded with a 6 lead ECG in normal (n=6), sham (n=10), MSCs (n=6), and the ccMSCs (n=7) groups (left panel) and representative examples of ECG in MSCs- and ccMSCs-engrafted rats (right panel). Multiform PVCs were frequent in the MSCs-engrafted rat, whereas no PVC was observed in the ccMSCs-engrafted rat. Values are the mean ± SD of at least three independent experiments. *$p<0.05$.

Supplemental Tables

Table S1. The expression profiles of inflammatory cytokines in the infarcted region.

Cytokines	Normal	Sham	MSCs	ccMSCs
IFN-γ	+	++	+	-
IL-1α	+	+++	++	+
IL-1β	+	+++	++	+
IL-6	+	+++	+++	++
TIMP-1	+	+++	++	+
TNF- α	+	++	+	+
VEGF	-	+	+	+++

Table S2. Baseline surface ECGs.

	Normal (n=6)	MI (n=12)	MSCs (n=9)	ccMSCs (n=12)
Heart rate (bpm)	278±57	323±56	350±58	334±58
RR(ms)	220±45	190±36	175±30	174±25
PR(ms)	46±8	46±11	50±12	49±12
QRS(ms)	45±4	62±16 *	59±7	46±7**,§
QTc(ms)	67±9	81±17Ŧ	80±24	66±20ŧ

QRS interval was significantly prolonged in sham group compared to normal (*;p<0.05). Slightly prolonged QRS duration in MSC engrafted rats were seen as compared to normal. The ccMSCs engraftment shortened QRS durations as compared to sham (§; <0.01) and MSCs (**;<0.05), respectively. Prolonged QTc interval was observed in sham compared to normal (Ŧ;<0.05), whereas QTc interval restored in ccMSCs group as compared to sham (ŧ;<0.05).